Short Communications

Effects of Hydrogen-Isotope Labelling on Steroid Metabolism

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Bolt *et al.* (1965) showed that *Botrodiploia malorum* readily converts 17β -hydroxyoestr-4-en-3-one into the corresponding 1β -hydroxy compound. We attempted to obtain 1β -hydroxyoestr-4-ene-3,17-dione by incubation of oestrenedione (oestr-4-ene-3,17-dione), but found that the major product was 7β -hydroxyoestrenedione (30–40% yield). A 10% yield of 6β -hydroxyoestrenedione was obtained whereas only a 3% yield of the corresponding 1β -hydroxy compound was realized. When the substrate was labelled at the C-7 β position with tritium and at C-4 with ^{14}C there was a marked increase in the $^3\text{H}/^{14}\text{C}$ ratio in the 6β -hydroxy compound. Similar results were obtained in competitive experiments with a mixture of unlabelled and 7β -deuterated oestrenedione. These results are interpreted to indicate that the hydrogen-isotope-labelled molecules were converted preferentially into 6β -hydroxyoestrenedione.

Incubations with steroids and extractions were carried out essentially as described by Bolt *et al.* (1965) with strain CBS 134.50 obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The extract was chromatographed on preparative-layer silica-gel plates with benzene–acetone mixtures. Zones corresponding in increasing order of polarity to oestrenedione, 6β -hydroxyoestrenedione and 7β -hydroxyoestrenedione were eluted and purified further by t.l.c. and crystallization. 1β -Hydroxyoestrenedione (Bolt *et al.* 1965; Townsley & Brodie, 1966) was separated from 7β -hydroxyoestrenedione (I) in the benzene–formamide paper system (R_{f} 0.67). 6β -Hydroxyoestrenedione was identified by comparison with material synthesized as described in the paper. The structure of 7β -hydroxyoestrenedione was indicated by the fact that it (m.p. 219–221°C) and its acetate (m.p. 189–191°C) formed oestra-4,6-diene-3,17-dione in excellent yield on mild treatment with 2% KOH in aqueous methanol at room temperature. 1-, 2-, 6- or 10-Hydroxylated 19-nor- Δ^4 -3-ketones do not have this property. The n.m.r. spectra for both compounds gave a signal for an axial hydrogen attached to a carbinol or carbinol acetate group (217 and 285 Hz respectively in C_2HCl_3 , broad multiplets, 1H; Varian A-60 instrument). Addi-

tional data (mass, i.r. and u.v. spectra, optical rotation and spectra in KOH and conc. H_2SO_4) also support this structure.

The labelled substrates were prepared by reduction of 17β -hydroxyoestra-4,6-dien-3-one (Turner & Ringold, 1967) with tris(triphenylphosphine)-rhodium(I) chloride catalysis (Djerassi & Gutzwiller, 1966) and tritium or deuterium gas. Both labelled products were purified by silica-gel and AgNO_3 –silica-gel t.l.c. with benzene–ethyl acetate (8:3, v/v) and chloroform–cyclohexane–acetic acid (6:3:1, by vol.) respectively. The 17β -hydroxy[6,7- ^3H]oestr-4-ene-3,17-dione product was diluted with 4 - ^{14}C -labelled material obtained by hydrolysis of 17β -acetoxy[4- ^{14}C]oestr-4-en-3-one (New England Nuclear Corp., Boston, Mass., U.S.A.). After equilibration with base (Brodie, 1967) to remove tritium from C-6, the $^3\text{H}/^{14}\text{C}$ ratio decreased by approx. 50%. [All radiochemical analyses were carried out by double-label scintillation counting (Townsley & Brodie, 1968).] The monotritiated product then was oxidized with Jones reagent (Bowers, Halsall, Jones & Lemm, 1953) to give [4- ^{14}C ,7- ^3H]oestrenedione without further change in the $^3\text{H}/^{14}\text{C}$ ratio. As additional indications of radiochemical purity, a portion of this material was converted into the $\Delta^{3,5}$ -enol ether (Kalvoda, Krähenbühl, Dessaulles & Anner, 1967) and then into 6β -hydroxyoestrenedione (Gardi & Lusignani, 1967), also without change in the $^3\text{H}/^{14}\text{C}$ ratio.

After incubation of [4- ^{14}C ,7- ^3H]oestrenedione [^3H (d.p.m.)/ ^{14}C (d.p.m.) ratio 28.6; ^{14}C specific radioactivity 2700 d.p.m./mg] with *B. malorum* for 21 h the isolated products from chromatography were diluted with appropriate amounts of non-radioactive materials. The isolated 7β -hydroxyoestrenedione after treatment with pyridine–acetic anhydride at room temperature and crystallization to constant specific radioactivity gave 7β -acetoxyoestrenedione with a $^3\text{H}/^{14}\text{C}$ ratio 0.45, representing over 98% loss in tritium radioactivity. This establishes that the tritium in the substrate was oriented over 98% β at C-7 since such hydroxylations occur by direct replacement of hydrogen (Hayano, 1962). 1β -Hydroxyoestrenedione, which

Table 1. *Incubation of deuterated oestrenedione with B. malorum*

Values, adjusted to the nearest whole unit, were obtained on a Varian M-66 mass spectrometer by averaging relative peak heights on scans from five or six atomic-mass-unit sweeps of the molecular-weight (M) or otherwise indicated range. Corrections for ^{13}C and ^{18}O content were made from analyses of non-deuterated standards; error, ± 3 absolute %. Conditions: electron energy, 70 V; current $50\mu\text{A}$; direct sample-injection probe temperature, $110\text{--}125^\circ\text{C}$.

	% of deuterated species		
	$^2\text{H}_0$	$^2\text{H}_1$	$^2\text{H}_2$
[6,7 β - ^2H]Oestrenedione*	3	4	93
[7 β - ^2H]Oestrenedione (substrate)†	67	34	0
7 β -Hydroxyoestrenedione (product)	100	0	0
6 β -Hydroxyoestrenedione (product)	43	56	1
6 β -Acetoxyoestrenedione	42	56	2
6 β -Acetoxyoestrenedione fragment ($M-42$)	44	55	2

* Undiluted synthesized material.

† Synthesized material base-equilibrated and diluted with non-labelled oestrenedione (see the text).

separated from the 7 β -hydroxy isomer on the benzene-formamide paper system, had a $^3\text{H}/^{14}\text{C}$ ratio very similar to that of the substrate. However, when 6 β -hydroxyoestrenedione was crystallized to constant specific radioactivity the $^3\text{H}/^{14}\text{C}$ ratio was 67, representing a 130% increase. The ratio became 72 on acetylation and crystallization and still remained high at 61 after oxidation of the alcohol with the Jones reagent to the 6-oxo compound. The latter result indicated that some exchange of label from C-7 occurred in this step. Treatment of the oxo compound with alkali removed the remaining label, also suggesting that in the 6 β -hydroxy product the label still was at C-7 β . The recovered substrate after an additional t.l.c. purification step had a $^3\text{H}/^{14}\text{C}$ ratio 25, which did not change on conversion into testosterone and testosterone acetate. This represents a 13% decrease in $^3\text{H}/^{14}\text{C}$ ratio and indicates there was no primary isotope effect operating during 7 β -hydroxylation.

A similar increase in label in the 6 β -hydroxy product was obtained with the 7 β -deuterated substrate. The [6,7 β - ^2H]oestrenedione material prepared was over 90% dideuterated and showed little evidence of random exchange (Table 1). The corresponding 7 β -monodeuterated material was prepared by base equilibration as above, and after crystallization was judged pure from the melting point, extinction coefficient at 240 nm, i.r. spectrum and chromatographic properties (including g.l.c. in QF-1 and SE-30 systems). This material, diluted with non-labelled recrystallized oestrenedione, was incubated for 15 h and the steroids then were extracted and purified as above. The 7 β -hydroxy- and 6 β -hydroxy-oestrenedione products were crystallized repeatedly until chromatographically pure and then were subjected to mass-spectral analysis.

As shown in Table 1, 7 β -hydroxyoestrenedione was devoid of deuterium whereas there was a 64% increase in the amount of deuterium in 6 β -hydroxyoestrenedione. After acetylation and crystallization the 6 β -acetate gave a similar analysis. In addition, precise deuterium analysis of the characteristic fragmentation-peak area near 288 ($M-42$) of the acetate spectrum gave values very close to those of the parent peak (Table 1).

The results indicate that the hydrogen-isotope-labelled compounds were 6 β -hydroxylated preferentially when made to react competitively with unlabelled or ^{14}C -labelled substrates. To our knowledge such an effect has not been noted previously in steroid metabolism. The purification and analytical steps followed make it unlikely that the results were influenced by the presence of impurities. Especially for the deuterium experiment, the agreement in analyses of the molecular-ion peak of the product, its acetate and the characteristic fragmentation peak of the latter is compelling evidence that the increased deuterium content is associated with the 6 β -hydroxy product. For the results to be caused by a heterogeneous substrate at least a 5–10% detectable impurity would have to be present and fortuitously be converted completely into the 6 β -hydroxy compound. In contrast only a 10% conversion of the logical precursor (substrate) was obtained. Oestr-5(10)-ene-3,17-dione, which would give the same molecular-ion-mass peak as the substrate, was readily excluded by the chromatographic purification steps used. As indicated above, the possibility of a primary isotope effect operating that would lead to enrichment of label in the substrate and subsequently in the 6 β -hydroxy compound has been eliminated. The possibility of isotope discrimination during chromatography can also be ruled out, since repeated

chromatography did not significantly alter the $^3\text{H}/^{14}\text{C}$ ratio in the radioactivity experiment.

It is not possible at this time to assign an explanation for the discrimination observed in the heterogeneous respiring culture system used. Oldham (1968) has pointed out, using as an example work done with alcohol dehydrogenase (Mahler & Douglas, 1957), that hydrogen-isotope-labelled substrates may be converted preferentially because of differences in binding constants to enzymes. There are also other examples of such differences, which may be quite large, as well as many reports of differences in chromatographic properties of such labelled compounds (for review see Simon & Palm, 1966). It is reasonable to suspect that the results reported here may be related to these binding phenomena.

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